Comparative Study of Bullous Pemphigoid Antigens Among Japanese, British, and U.S. Patients Indicates Similar Antigen Profiles with the 170-kD Antigen Present both in the Basement Membrane and on the Keratinocyte Cell Membrane

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There are a number of controversies relating to studies of bullous pemphigoid (BP) antigens from different institutions, mainly regarding the detection of the 230-kD and 170-kD BP antigens. In this study, in an attempt to resolve the discrepancies, we have examined and compared the reactivity by immunofluorescence, immunoblotting, and immunoprecipitation among the sera from Japanese, British and U.S. BP patients. Both the 230-kD and 170-kD BP antigens were detected by various sera from all populations with immunoblotting, whereas immunoprecipitation detected only the 230-kD BP antigen but not the 170-kD BP antigen. Immunoprecipitation was more sensitive than immunoblotting to detect the 230-kD antigen. These results indicate that both the 230-kD and 170-kD proteins are BP antigens found in all three populations. By immunofluorescence cell surface staining in the lower epidermis in addition to basement membrane zone staining was shown by a considerable number of patients' sera in all populations. Comparison between the results of immunofluorescence and immunoblotting revealed a clear correlation of this cell surface staining with the presence of antibodies against the 170-kD BP antigen. That the affinity-purified antibodies specific to the 170-kD BP antigen showed this cell surface staining further confirmed this correlation. These results may indicate a different nature of the 170-kD BP antigen from that of the 230-kD BP antigen. *J Invest Dermatol 100:385–389, 1993*

The presence of anti-epidermal basement membrane zone (BMZ) autoantibodies is now a hallmark in the diagnosis of bullous pemphigoid (BP) [1,2]. The BP antigens that are defined immunologically by anti-BMZ antibodies have been extensively investigated [3–24], although there are still some controversies in studies from different institutions. Some studies have suggested that BP antibodies recognize a single antigen [3–7], whereas others have indicated that there may be molecular heterogeneity in BP antigens [8–17]. In general, BP antigen was identified as a unique 230-kD protein by immunoprecipitation [3–6]. However, immunoblot analysis showed several different BP antigens, mainly the 230-kD and 170-kD proteins [9–17]. Furthermore, the frequency of detection of the 230-kD and 170-kD BP antigens is considerably different among differing studies [9–17]. These discrepancies may be due to the different techniques used. Another possibility is that there may be different subsets of BP patients in differing populations.

To clarify these discrepancies, we examined the sera from patients in different population groups by immunofluorescence, immunoblot, and immunoprecipitation assays. No obvious difference was found in the results for each group of patients. Furthermore, the correlation of the immunofluorescence results with those of immunoblotting indicated the possibility that the antibodies against the 170-kD BP antigen may be responsible for the cell surface staining in the lower epidermis.

Manuscript received February 7, 1992; accepted for publication December 12, 1992.

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Abbreviations:
BMZ: basement membrane zone
BP: bullous pemphigoid
BSA: bovine serum albumin
EDTA: ethylenediaminetetraacetic acid
PMSF: phenylmethylesulfonyl fluoride
SDS-PAGE: sodium dodecylsulfate–polyacrylamide gel electrophoresis
TBS: Tris-HCl--buffered saline, pH 8.0

MATERIALS AND METHODS

Sera Sera were obtained from 35 Japanese BP patients, 29 British BP patients, 11 U.S. BP patients, and 10 normals. All sera were stored at −20°C or stored at 4°C in the presence of 0.1% NaN_3 for less than 1 month during these experiments.

Immunofluorescence Indirect immunofluorescence was performed by standard method [1] using normal human skin section as a substrate. To remove the antibodies against blood type antigens, some BP sera were pre-absorbed with an eightfold volume of red blood cell pellet of AB type at room temperature for 2 h before immunofluorescence.

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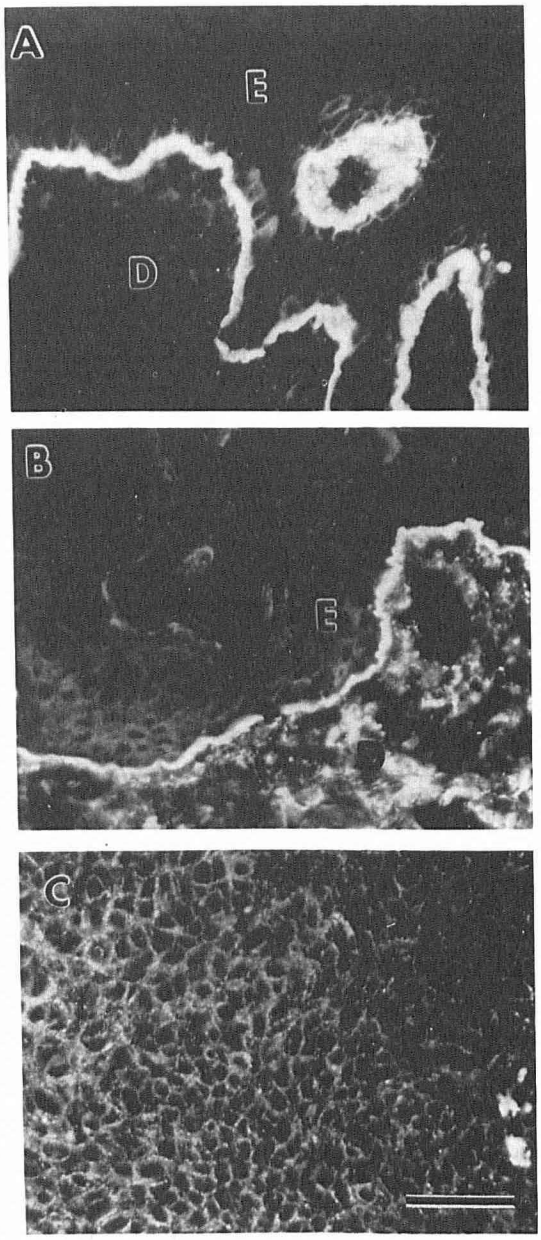

Figure 1. Results of immunofluorescence. *A*: The serum of U.S. BP patient A5 that reacted only with the 170-kD BP antigen with immunoblotting stained the cell surface in the lower epidermis as well as the BMZ (magnification ×400). E and D, epidermis and dermis, respectively. *B*: The serum of British BP patient B1 reactive only with the 230-kD BP antigen stained only the BMZ (magnification ×400). *C*: The serum of A5 also stained the cell surface of the tumor cells of hydroacanthoma simplex (magnification ×400). *Bar*, 10 μm.

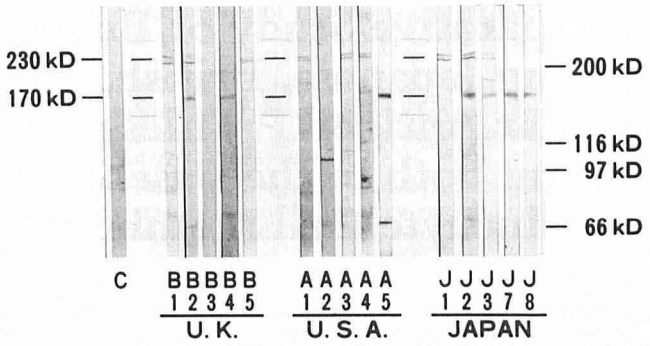

Figure 2. Results of immunoblot analyses. The reactivity of five representative BP sera each of U.K. *(lanes B1–B5)*, U.S. *(lanes A1–A5)*, and Japan (*lanes J1–J3, J7,* and *J8*), and one normal serum *(lane C)* is depicted. The number shown beneath each lane corresponds to the same number in Fig 3. The positions of the 230-kD and 170-kD BP antigens are shown to the left. The migrations of standard molecular markers are shown to the right. The positions of the both BP antigens in each set of five lanes are connected by *horizontal bars*. Most BP sera reacted with the 230-kD and/or the 170-kD BP antigens in a variety of patterns. The 230-kD BP antigen was detected as a doublet of protein bands in this particular experiment.

Immunoblot Analysis Immunoblots with ethylenediaminetetraacetic acid (EDTA)–separated normal human epidermal extracts were performed as described previously [10].

Immunoprecipitation Immunoprecipitation was performed mainly according to the method described previously [3]. We used KU8 cells, a human squamous cell carcinoma cell line originating from a penile carcinoma [25]. KU8 cells were radiolabeled with ^{35}S-methionine (0.925×10^6 Bq/ml) in methionine-free medium containing 10% fetal calf serum, and lysed with 0.5% Nonidet P-40 in the Tris-HCl–buffered saline (TBS, pH 8.0) supplemented with 2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 10,000 × *g* at 4°C for 40 min, the supernatant was dialyzed against TBS containing 0.3% Nonidet P-40 and 2 mM PMSF overnight, and stored at −80°C as aliquots. To reduce nonspecific background, a 200-μl antigen sample ($2.4–3.6 \times 10^7$ cpm) was first pre-absorbed with 20 μl of combined sera from three normal subjects at 4°C overnight, and then precipitated by incubation with 60 μl of Protein G Sepharose slurry (Pharmacia, Uppsala, Sweden) at 4°C for 30 min. Fifteen microliters pre-absorbed antigen sample was incubated with 3 μl of either BP or normal sera at 4°C overnight. Antigen-antibody complexes were precipitated by incubation with 3 μl Protein G Sepharose slurry at 4°C for 30 min. After the precipitates were washed six times with TBS containing 0.3% Nonidet P-40, 0.3% sodium deoxycholate, and 2 mM PMSF, Protein G, IgG, and antigens were dissociated and reduced by boiling in Laemmli's sample buffer (0.00625 M Tris-HCl, pH 6.8, containing 2% sodium dodecylsulfate (SDS) and 5% β-mercaptoethanol), and Protein G was removed by centrifugation. After proteins were fractionated by SDS–polyacrylamide gel electrophoresis (PAGE), gels were processed for fluorography.

Affinity-Purification of Autoantibodies According to the previously reported method [16], autoantibodies were affinity purified from nitrocellulose membrane. Briefly, horizontal strips containing appropriate antigens were cut from membrane blotted with SDS-PAGE fractionated epidermal extracts, blocked with 3% skim milk in TBS, incubated with a BP serum and extensively washed. Bound antibodies were eluted for 30 min at 37°C with 20 mM sodium citrate (pH 3.2) containing 0.5% bovine serum albumin (BSA) and 0.05% Tween 20, neutralized with 2 M Tris-HCl (pH 7.5), dialyzed against TBS, concentrated, and subjected to immunofluorescence. Another horizontal strip from unrelated area of the same

Table I. Summary of the Results of Immunoblotting

Immunoblot	Japan	U.K.	U.S.A.	Total
230 kD only (+)	10	10	8	28
170 kD only (+)	9	8	2	19
Both (+)	16	7	0	23
Both (−)	0	4	1	5
Total	35	29	11	75

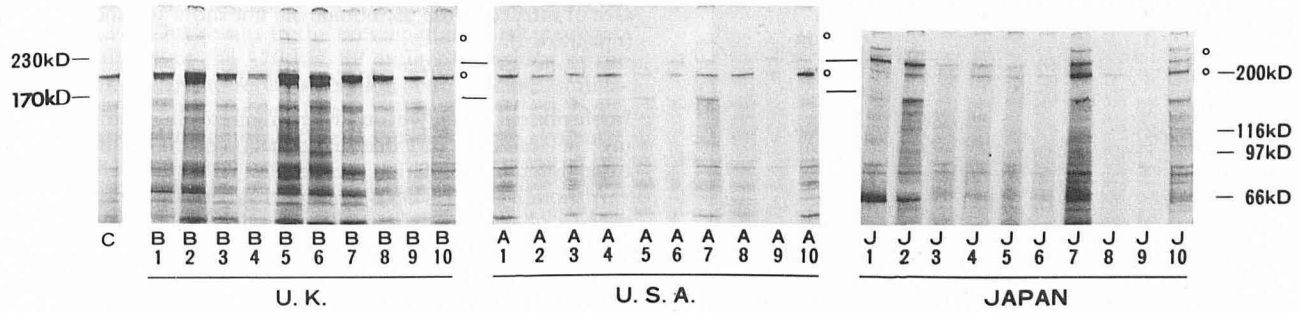

Figure 3. Results of immunoprecipitation for 10 representative BP sera each of three population groups. The position of the 230-kD BP antigen and the level where the 170-kD BP antigen is supposed to migrate are shown to the left. The migrations of standard molecular markers are shown to the right. The positions of the 230-kD and 170-kD BP antigens are connected by *horizontal bars*. Two protein bands of high molecular weight and approximately 200 kD (shown by *open circles* to the right of each set of 10 lanes) constantly seen in our immunoprecipitation were considered non-specific, because the protein bands were seen even in normal control serum *(lane C)*. The 230-kD BP antigen was detected by most BP sera, although any sera showed no specific protein band corresponding to the 170-kD protein.

nitrocellulose membrane was similarly processed, and the eluate was used as a negative control.

RESULTS

Immunofluorescence With immunofluorescence of normal human skin sections, all patients' sera showed anti-BMZ antibodies at a titer of 1:20 or more. Furthermore, reactivity with the cell surface in the lower epidermis was also seen in the sera of 23 BP patients (15 of 35 Japanese patients, six of 29 British patients, and two of 11 U.S. patients). The intensity of the reactivity was variable. Although 18 sera reacted with only the lateral or entire cell surface of basal cells, five sera showed the cell surface staining in the two to three cell layers of the lower epidermis (Fig 1*A,B*). Because the cell surface staining seemed to be restricted to the lower undifferentiated cells in the epidermis, we also used cryostat sections from a case of hydroacanthoma simplex, a benign skin tumor derived from intraepidermal eccrine sweat duct, in which tumor cells show similar appearance to basal cells. When this section was used as a substrate, 12 of these 23 BP sera showed strong reactivity with the cell surface of all tumor cells (Fig 1*C*). To rule out the possibility that this cell surface staining was due to anti-blood type antibodies, we pre-absorbed these sera with red blood cells of type AB before immunofluorescence. No reduction of the cell surface reactivity was seen in any sera after the pre-absorption. Some sera showed cytoplasmic staining of basal cells, which was easily distinguished from the cell surface staining by its different staining pattern.

Immunoblot Analysis With immunoblotting, most BP sera labeled the two known BP antigens, i.e., the 230-kD and 170-kD proteins, in a variety of patterns (Fig 2 and Table I). Both the 230-kD and 170-kD BP antigens were detected by sera of BP patients in all three populations. However, an apparent difference noted was that although the 170-kD BP antigen was detected by a majority of the sera from Japanese and British patients, only two of 11 sera reacted with the 170-kD BP antigen in U.S. patients. The patterns of reactivity were otherwise almost identical among the three populations. Ten control sera did not show either of these protein bands.

Immunoprecipitation All sera from Japanese and U.S. BP patients and 16 of 29 British BP patients were examined by immunoprecipitation. Forty-seven of 62 tested sera precipitated the 230-kD BP antigen (Fig 3). However, none of the patients' sera precipitated the protein corresponding to the 170-kD BP antigen. None of the seven control sera that were not used for pre-clearance of the cell extracts showed any specific reactivity either. The correlation of detection of the 230-kD BP antigen between immunoblotting and immunoprecipitation is summarized in Table II. All 43 BP sera that detected the 230-kD BP antigen with immunoblotting also immunoprecipitated the 230-kD protein. However, four of 19 BP sera that did not detect the 230-kD BP antigen by immunoblotting did immunoprecipitate the 230-kD protein, although the remaining 15 sera did not detect the 230-kD protein even with immunoprecipitation.

Relationship Between the Results of Immunofluorescence and Immunoblotting Table III summarizes the relation of the pattern of reactivity with immunoblotting to the cell surface staining with immunofluorescence. Surprisingly, all the 23 sera that showed the cell surface staining with immunofluorescence detected the 170-kD BP antigen with immunoblotting, although the remaining 19 sera reactive with the 170-kD protein did not show the cell surface staining. By contrast, any sera that did not detect the

Table II. Relationship of Detection of the 230-kD BP Antigen Between Immunoblotting and Immunoprecipitation

Immunoblot	Immunoprecipitation	Japan	U.K.	U.S.A.	Total
230 kD (+)	230 kD (+)	26	9	8	43
	230 kD (−)	0	0	0	0
230 kD (−)	230 kD (+)	0	2	2	4
	230 kD (−)	9	5	1	15
Total		35	16	11	62

Table III. Relationship Between the Results in Immunoblotting and the Cell Surface Staining with Immunofluorescence

Immunoblot	Cell Surface Staining with Immunofluorescence	Japan	U.K.	U.S.A.	Total
230 kD only (+)	(+)	0	0	0	0
	(−)	10	10	8	28
170 kD only (+)	(+)	7	6	2	15
	(−)	2	2	0	4
Both (+)	(+)	8	0	0	8
	(−)	8	7	0	15
Both (−)	(+)	0	0	0	0
	(−)	0	4	1	5
Total		35	29	11	75

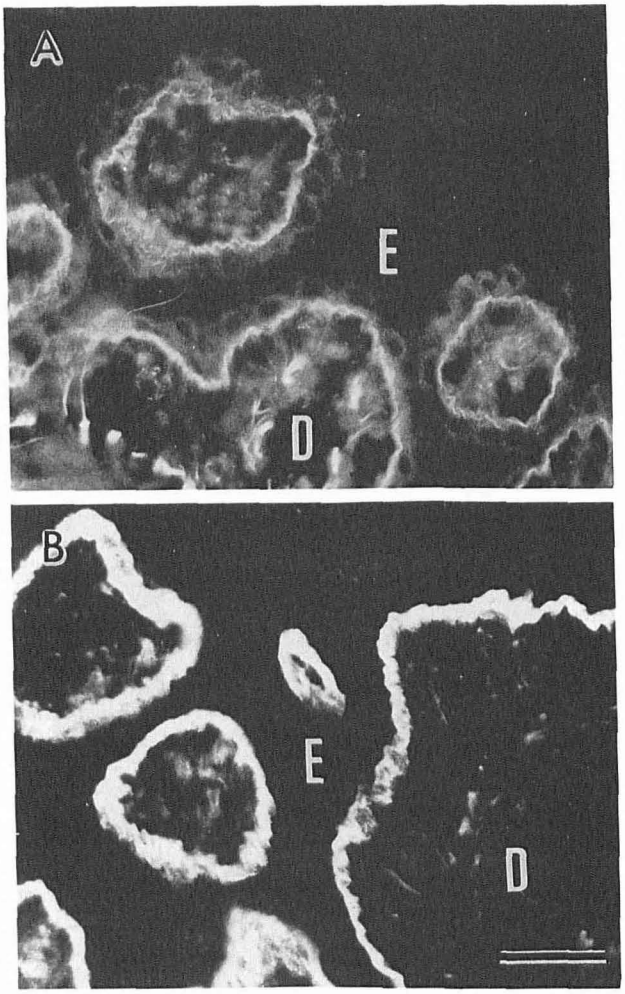

Figure 4. Reactivity of affinity-purified antibodies on immunofluorescence. *A*: The eluate from the 170-kD BP antigen stained the cell surface in the lower epidermis as well as the BMZ (magnification ×400). E and D, epidermis and dermis, respectively. *B*: The eluate from the 230-kD BP antigen showed only the BMZ staining (magnification ×400). *Bar*, 10 μm.

170-kD BP antigen with immunoblotting did not show the cell surface staining. The cell surface staining was shown by as many as 15 of 19 sera that reacted only with the 170-kD protein, whereas only eight of 23 sera that reacted with both the 230-kD and 170-kD proteins showed the cell surface staining. This tendency was almost the same among the three populations. There is no correlation between the immunofluorescence titer of sera and the cell surface staining. Some BP sera with low titer (around 1 : 40) showed the cell surface staining, and many sera with high titer (> 1 : 640) did not show the staining.

Reactivity of Affinity-Purified Antibodies From a Japanese BP patient's serum ("J2" shown in Fig 2 and Fig 3) that clearly stained the cell surface in the lower epidermis with immunofluorescence and labeled both the 230-kD and 170-kD BP antigens with immunoblotting, autoantibodies specific either to the 230-kD BP antigen or to the 170-kD BP antigen were affinity purified and examined by immunofluorescence. The eluate from the 170-kD BP antigen stained the cell surface in the lower epidermis as well as the BMZ (Fig 4*A*). In contrast, the eluate from the 230-kD BP antigen showed only the BMZ staining (Fig 4*B*). The eluate from unrelated area of nitrocellulose membrane did not show any specific staining (not shown).

DISCUSSION

In the present study, we found several interesting results by comparing the reactivity in three different assays among BP patients of three different population groups.

Although the 170-kD BP antigen has been frequently detected in many studies [9–17], some reports, mainly from U.S. investigators, include no description for the 170-kD BP antigen, even with immunoblot studies [3–7]. In this study, we found the 170-kD BP antigen–positive sera in all groups of BP patients including U.S., indicating that the 170-kD protein is one of the BP antigens in all tested populations. The lack of reactivity of the 170-kD BP antigen by immunoblotting from some institutions may be due to differences in the techniques used. One possible reason is that proteinase inhibitors may not be adequate, if the 170-kD BP antigen is particularly sensitive to proteinase.

Although immunoblot assay has clearly demonstrated the 170-kD BP antigen [9–17], the 170-kD protein has not been reported to be detectable with immunoprecipitation [3–6]. In this study, we could not detect the 170-kD protein with our immunoprecipitation, even with the BP sera that showed strong reactivity with the 170-kD protein by immunoblotting. It has been reported that a faint 166-kD protein band was occasionally seen with immunoprecipitation [8]. This 166-kD protein may be the same as the 170-kD BP antigen. However, because this protein was always associated with the more prominent 230-kD protein, the 166-kD protein may also be a degradation product of the 230-kD protein but not the 170-kD BP antigen. The reason why the 170-kD BP antigen is not detected by immunoprecipitation is at present unknown.

A previous study reported that immunoprecipitation is more sensitive than immunoblotting [8]. In that study, the 230-kD BP antigen was detected by almost all BP sera, whereas some sera did not detect the 230-kD protein by immunoblotting. In the present study, we also found that all BP sera reactive with the 230-kD BP antigen with immunoblotting immunoprecipitated the same protein and, furthermore, some sera negative for the 230-kD protein with immunoblotting clearly demonstrated the antigen with immunoprecipitation. This confirms that immunoprecipitation is more sensitive than immunoblotting for detection of the 230-kD BP antigen.

In general, the present study indicates that there is similar reactivity of BP sera among the three different population groups. The discrepancies found in previous reports are most likely to be due to the different techniques used in individual institutions. This indicates that the use of standardized techniques among different laboratories may be required for future progress in the investigation of BP antigens.

A surprising result obtained during this investigation was a close relationship between the cell surface staining with immunofluorescence and the presence of the antibodies to the 170-kD BP antigen. We previously noticed that a few BP sera react with the cell surface in the lower epidermis as well as the BMZ [26,27]. In the present study, we found 23 of 75 BP sera demonstrated the cell surface staining in the lower epidermis. All the sera showing the cell surface staining detected the 170-kD BP antigen by immunoblotting, whereas the cell surface staining was not shown by any sera negative for the 170-kD BP antigen. These results strongly suggest that antibodies against the 170-kD BP antigen are responsible for the cell surface staining. This speculation was further confirmed by the results of the study using affinity-purified antibodies that the antibodies specific to the 170-kD BP antigen stained the cell surface in the lower epidermis as well as the BMZ, whereas the antibodies specific to the 230-kD BP antigen showed only the BMZ staining.

Most BP sera reactive only with the 170-kD BP antigen showed the cell surface staining, whereas fewer of the sera reactive with both the 230-kD and 170-kD BP antigens showed this pattern of staining. The reason for this is at present unknown.

Recently, cDNA cloning indicated that the 170-kD BP antigen

has a quite unique structure with the characteristics of a collagen molecule [23,24]. Our results in the present study further suggest the different nature of the 170-kD BP antigen from that of the 230-kD BP antigen. In future, consideration of the possible localization of the 170-kD BP antigen in the cell surface of the lower epidermis may give some insights for the studies of this protein.

We are grateful to Ms. K. Nakane for the excellent technical assistance. This study was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (02557045); a joint research grant of Monbusho International Scientific Research Programme, Japan (02044130); a collaboration research project of the British Council, Tokyo, Japan; and a grant from the Ministry of Health and Welfare of Japan.

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